Cytoprotective effects of ferritin on doxorubicin-induced breast cancer cell death

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Abstract. Ferritin is a major iron storage protein and essential for iron homeostasis. It has a wide range of functions in the body including iron delivery, immunosuppression, angiogenesis, and cell proliferation. Ferritin is overexpressed in many cancer cells, but its precise role in cancer is unclear. In the present study, we examined the functional roles of ferritin in protecting the MCF-7 breast cancer cell line against treatment with the chemotherapeutic agent doxorubicin. The effects of ferritin (human liver ferritin) and doxorubicin on the human MCF-7 breast cancer cell line were evaluated using the cell viability assay. The impact of decreasing ferritin light chain (FTL) and ferritin heavy chain (FTH) expression on doxorubicin sensitivity was assessed using siRNA. Reactive oxygen species (ROS) was also measured using the fluorescence probe CM-H_2DCFDA. The mechanism of modulated chemosensitivity was evaluated by western blot analysis. Ferritin treatment activated MCF-7 cell proliferation in a concentration- and time-dependent manner. While treatment with doxorubicin alone significantly increased intracelullar ROS production, the addition of ferritin decreased this ROS formation, thereby reducing doxorubicin-induced MCF-7 cell death. The inhibition of FTL and FTH by siRNA sensitized cells to doxorubicin. Treatment with doxorubicin alone significantly induced the cell cycle-dependent kinase inhibitor protein p21, whereas ferritin reduced p21 expression. Thus, ferritin plays a critical role in protecting MCF-7 cells against the chemotherapeutic drug doxorubicin. A targeted reduction of ferritin may be a useful strategy for overcoming chemoresistance in breast cancer.

Introduction

Worldwide, breast cancer is the most common malignancy in women and also the leading cause of cancer mortality. Resection of tumors with or without adjunctive chemotherapy is the only00000 known strategy for long-term survival. More than 50% of breast cancer cases are either intrinsically resistant or rapidly acquire resistance to various anticancer drugs (1). Currently, drug resistance or drug inefficacy are major obstacles in the successful treatment of breast cancer (2). New chemotherapeutic strategies are therefore needed.

Ferritin is a protein that binds to and stores iron. It is composed of two subunits, the ferritin light chain (FTL, L-subunit, 19 kDa) and the ferritin heavy chain (FTH, H-subunit 21 kDa) (3,4). Ferritin serves as a critical component of iron homeostasis and requires the participation of the two subunits (5-8). The H-subunit has ferroxidase activity, it is essential and sufficient for rapid iron uptake (9), while the L-subunit facilitates stable iron storage in the ferritin core (5). Recent findings have shown that ferritin is involved in cell proliferation, angiogenesis, immunosuppression, iron delivery and iron storage (10-13). Elevated serum and tissue ferritin are associated with cancer (14) and may contribute to cancer cell survival and drug resistance.

Ferritin is detected in the serum of cancer patients, with higher levels correlating with more aggressive disease and poorer clinical outcomes. It is differentially overexpressed in several malignancies including breast cancer, liver cancer, lymphoma, and pancreatic cancer (15). In breast tumor tissues, L-ferritin levels are ~6-fold higher than surrounding benign breast tissue (10,16,17). This increase correlates with greater epithelial cell proliferation, histopathological dedifferentiation, shorter survival rate and chemotherapeutic resistance (18). Alkhateeb *et al* have shown that ferritin stimulates proliferation of the MCF-7 and T47D breast cancer cell lines (10).

The source of serum ferritin remains to be determined. Previously conducted studies have indicated that ferritin is generated and secreted from tumor-associated macrophages (TAMs) (10,16). The high levels of ferritin in TAMs may protect them from iron-induced damage, stimulate survival, proliferation, and angiogenesis (11). Moreover, the secretion of ferritin from TAMs may have a direct role in promoting and maintaining tumor proliferation.

It has recently been shown that the repression of ferritin by siRNA increases the chemosensitivity of HeLa cells (11) and human glioma (19). Of note, the downregulation of ferritin by miR-200b is also associated with an increased sensitivity of the MDA-MB-231 breast cancer cell line to doxorubicin (20).

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These results suggested that ferritin may be an attractive target for cancer treatment because its suppression may induce cancer cell death and increase the efficacy of chemotherapy.

However, to the best of our knowledge, there have been few reports on the effects of ferritin in breast cancer cells. In the present study, we investigated whether ferritin confers protection against chemotherapeutic drugs in the MCF-7 breast cancer cell line. We hypothesized that the inhibition of ferritin was able to sensitize MCF-7 cells to doxorubicin and may be a viable strategy for improving the efficacy of other anticancer drugs against breast cancer.

Materials and methods

Reagents. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and other cell culture ingredients were purchased from Life Technologies (Grand Island, NY, USA). PCR array ingredients were supplied by SABiosciences (Frederick, MD, USA). Human liver ferritin was obtained from EMD (Darmstadt, Germany) and doxorubicin from Sigma Co. (St. Louis, MO, USA).

Cell viability assay. The human breast cancer cell line MCF-7 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained according to the manufacturer's instructions at 37˚C and 5% CO_2 in DMEM (Gibco, Grand Island, NY, USA). DMEM was supplemented with 10% FBS and renewed every 3 days, trypsinized with 0.05% trypsin-EDTA and subcultured in the same medium.

The effects of human liver ferritin and doxorubicin on the MCF-7 cell line were then determined. Briefly, MCF-7 cells were seeded into 96-well culture plates at a density of 2.5×10^4 cells/well for 24 h, and cells were starved with FBS-free medium for 24 h. Non-complete fresh media containing test compound, human liver ferritin and doxorubicin were then incubated at the indicated times. Cytotoxicity was measured using the CellTiter 96 AQueous Cell Proliferation Assay kit (MTS; Promega, Madison, WI, USA) and MTT Cell Proliferation kit I (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions.

Transient transfection of FTL and/or FTH small-interfering RNA. Pre-designed FTL siRNA (siGENOME SMARTpool siRNA no. E-016214-00-0005) and control siRNA (siGENOME non-targeting siRNA no. D-001210-02-20) were purchased from Thermo Scientific (Waltham, MA, USA). FTH siRNA (sc-40575) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

The cells were grown in a 6-well plate to a confluence of 70%. For each well, 100 nM of FTL siRNA and 400 nM of FTH siRNA were mixed with 2 μl of Lipofectamine 2000 and 1 ml of OptiMEM® medium was added. The cells were exposed to the transfection mixture for 4 h. At the end of the incubation period, the medium was discarded, 2 ml of antibiotic-free DMEM complete medium was added, and the cells were cultured for an additional 20 h. The siGENOME non-targeting siRNA (D-001210-02-05; Dharmacon) was used as a negative control, and was introduced to the cells using the same protocol. Total RNA was extracted from MCF-7 cells after transfection. Efficiency of the transient transfection was determined by reverse transcription-polymerase chain reaction (RT-PCR) using TaqMan® primers.

To assess the cytotoxicity of doxorubicin against the FTL and FTH knockdown of MCF-7 cells, MCF-7 cells (2.5×10^4 cells/well) were grown in a 96-well plate for 24 h. For each well, cells transfected with 3 nM of FTL siRNA and 12 nM of FTH siRNA reagent were mixed with 0.06 μl of Lipofectamine 2000 in OptiMEM® medium. The cells were kept in the transfection mixture for 4 h. Subsequently, 100 μl of antibiotic-free DMEM complete medium were added and the cells were maintained for an additional 20 h. The cells were treated with 1 μM doxorubicin for an addtional 24 h prior to performing the cell viability assay as described above.

Intracellular reactive oxygen species formation. Intracellular reactive oxygen species (ROS) generation was measured using the cell-permeable fluorescent probe, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA). MCF-7 cells (2.5×10^4 cells/well) were cultured in 96-well plates for 24 h. Treatment with human liver ferritin and doxorubicin was performed, alone and in combination with 10 μM CM-H_2DCFDA for 90 min at 37˚C in the dark. DCF fluorescence was measured at 488 (excitation) and 520 nm (emission) on a fluorescence microplate reader. The data were presented as the percentage of ROS relative to the untreated controls.

Reverse transcription-polymerase chain reaction. The MCF-7 cells (1.5×10^6 cells/well) were seeded in 6-well plates and allowed to grow for 24 h. Subsequently, the cells were treated with the test compounds and RNA was isolated using the RNeasy Mini kit according to the manufacturer's instructions. Recovered RNA was quantified by its absorbance at 260 nm. cDNA was prepared by reverse transcription of isolated RNA using the RT^2 First Strand kit. The reverse transcription products served as a template for RT-PCR. RT-PCR was carried out according to the TaqMan® manufacturer's instructions (code number for FTL: Hs00830226_gH, FTH: Hs01060665_g1, and β-actin: Hs01694011_s1, internal control). The expression of each gene was monitored using an ABI 7900 (Applied Biosystems, Bedford, MA, USA). Gene expression difference levels were calculated using the $2^{-\Delta\Delta Ct}$ method for relative quantification and expressed as the fold change relative to the untreated control. Data were normalized to the expression of messenger RNA for β-actin, included on the same polymerase chain reaction array plate with targeted genes.

Western blot analysis. Western blot analysis was used to determine the expression levels of FTL, p21 and the internal control β-actin. MCF-7 cells were cultured in 6-well plates and treated with human liver ferritin, doxorubicin or a combination of the two test compounds at the indicated times. The cultured cells were washed with PBS, lysed with RIPA buffer (Sigma Co.) and protease inhibitor cocktail (1:100, M221; Amresco, Solon, OH, USA) at 4˚C for 30 min prior to being transferred to a microcentrifuge tube. After vigorous vortex mixing, the suspension was centrifuged at 12,000 x g for 30 min and the supernatant was collected and stored at -70˚C until use. Total protein concentration was determined using Pierce BCA Protein Assay kit (Thermo Scientific).

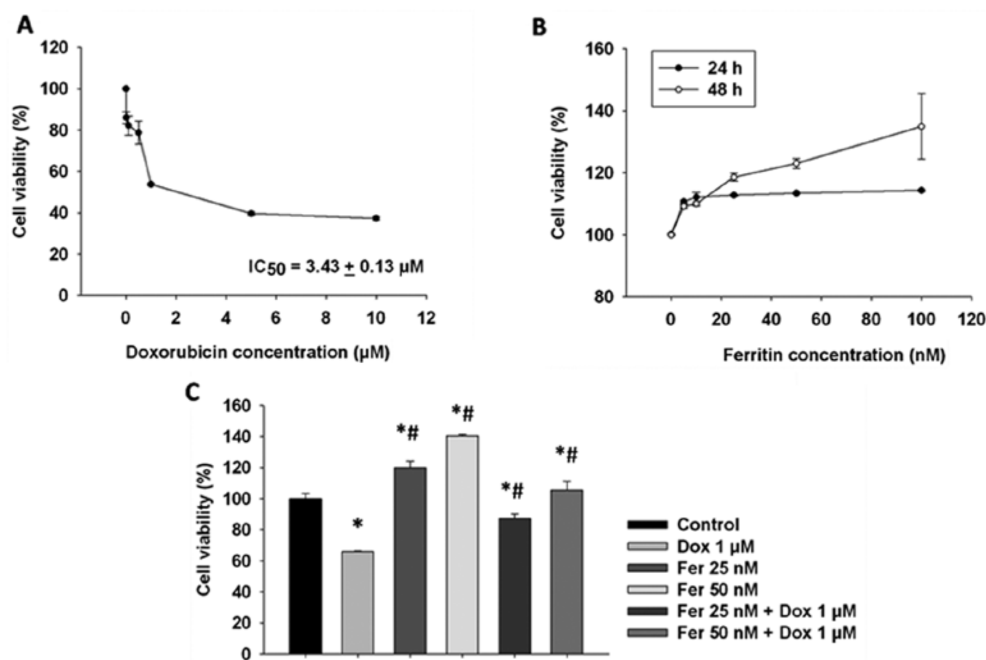

Figure 1. Effect of ferritin and doxorubicin on cell viability. MCF-7 cells were seeded in 96-well culture plates for 24 h and then starved with FBS-free medium for 24 h. Cells were treated with various concentrations of human liver ferritin (5-100 nM) for 24 and 48 h (A), and various concentrations of doxorubicin (0.01-10 μM) for 24 h (B). On day 1 of the combination treatment, cells were starved with FBS-free DMEM for 24 h. On day 2, the cells were exposed to human liver ferritin (25 and 50 nM) for 24 h (C). On day 3, the cells were exposed to doxorubicin (1 μM) for a further 24 h. After treatment, cell viability was determined by the MTS and MTT assays. The bars show mean ± SEM, each from three independent experiments. *P<0.05 vs. untreated control groups. #P<0.05 vs. doxorubicin groups.

The protein samples were mixed with SDS loading buffer and subjected to separation by electrophoresis in 4-20% Criterion polyacrylamide Tris-HCl gel (Bio-Rad, Hercules, CA, USA). The bands were blotted onto a nitrocellulose membrane. The membranes were blocked for 1 h at room temperature with 5% (w/v) skimmed milk powder in Tris-buffered saline (TBS) containing 0.1% Tween-20. The membranes were then incubated overnight at 4˚C with primary antibodies of rabbit polyclonal anti-human FTL (1:1,000, ab69096), rabbit polyclonal anti-human p21Cip/WAF1 (1:500, ab7960), and rabbit polyclonal anti-human β-actin (1:2,500, ab8227) (all from Abcam, Cambridge, USA) in TBS. After washing with TBS, the blots were incubated for 1 h at room temperature with the HRP-conjugated secondary antibodies (anti-rabbit IgG-HRP, 1:5,000, ab97051; Abcam). After removal of the secondary antibody and TBS buffer washes, the blots were incubated in chemiluminescent (ECL) system (Perkin Elmer, Waltham, MA, USA). The densities of the specific protein bands were visualized and captured by Multi Gauge software (V3.0; FujiFilm Systems Medical Systems, Stamford, CT, USA).

Statistical analysis. Data are presnted as mean ± SEM of duplicate assays from three independent experiments. An analysis of variance with repeated measurement was used to determine significant differences between each experimental group. The level of significance was set at P<0.05.

Results

Ferritin and doxorubicin effects on MCF-7 cell viability. To assess the proliferative roles of ferritin with or without doxorubicin in MCF-7 cells, the cells were exposed to ferritin and doxorubicin and assessed for viability by MTS and MTT. Doxorubicin reduced cell viability in a dose-dependent manner with IC_{50} values of 3.43±0.13 μM at 24 h (Fig. 1A). Ferritin, by contrast, induced cell proliferation with maximal induction observed after 24-48 h (Fig. 1B). When MCF-7 cells were treated with ferritin (25 and 50 nM) for 24 h the cytotoxic effect of doxorubicin was significantly decreased (Fig. 1C).

Ferritin and doxorubicin effects on FTL expression in MCF-7 cells. To establish whether ferritin and doxorubicin was capable of inducing FTL expression in MCF-7, cells were incubated with 25 nM ferritin and 1 μM doxorubicin and the time-course of FTL expression was determined by western blotting and RT-PCR. Ferritin-treated MCF-7 cells showed rapidly elevated FTL protein within 6 h compared with the control, although FTL expression was unaltered (Fig. 2A). The combined treatment significantly increased levels of FTL protein compared to the control and ferritin treatment alone (Fig. 2B). FTL mRNA expression was unaltered by ferritin in the presence or absence of doxorubicin.

FTL and FTH gene silencing sensitizes MCF-7 cells to doxorubicin. To confirm that FTL and FTH inhibition induced the sensitization of MCF-7 cells to anticancer agents, we examined the effects of FTL and FTH gene silencing on doxorubicin sensitivity. Basal levels of FTL and FTH were determined by RT-PCR. FTL mRNA expression was found to be higher than that of FTH (Fig. 3A). FTL and FTH mRNA significantly decreased 24 h after transfection of siRNA, respectively (Fig. 3B and C). At 24 h after siRNA treatment,

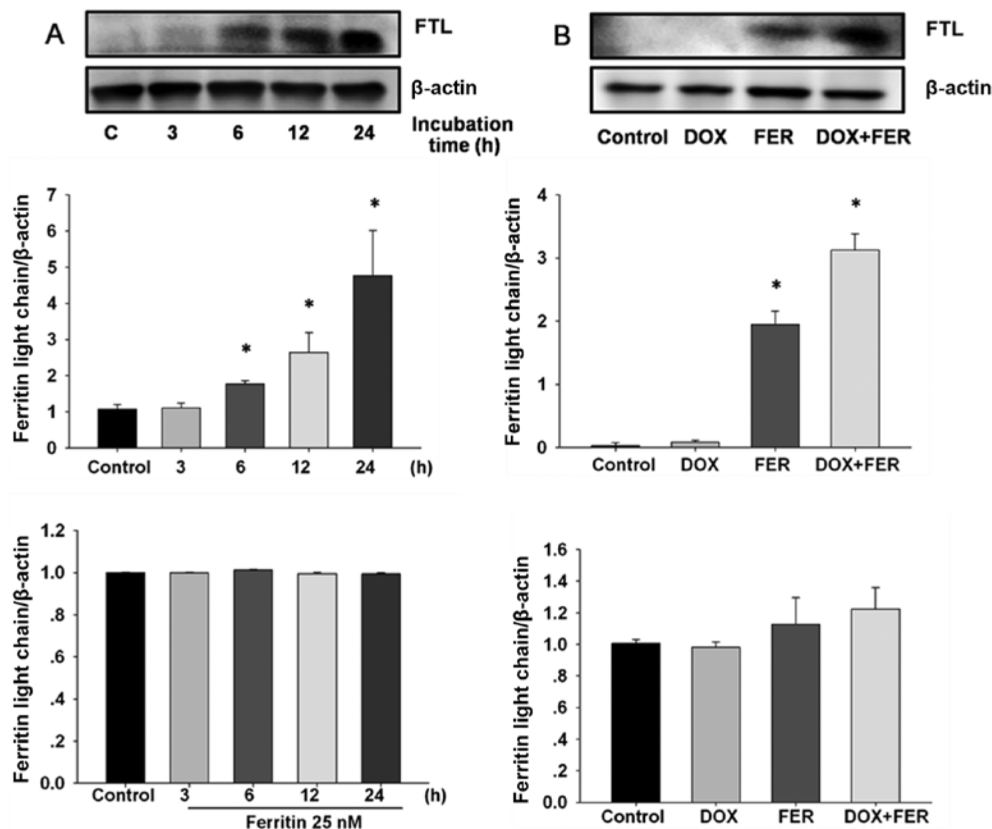

Figure 2. Effects of ferritin and doxorubicin on FTL expression in MCF-7. Cells were seeded in a 6-well culture plate for 24 h and starved for 24 h. The cells were treated with 25 nM human liver ferritin for 3-24 h (A). On day 1 of the combination treatment, the cells were pretreated with 25 nM human liver ferritin for 24 h, followed by 1 μM doxorubicin for 24 h on day 2 (B). The cytosol protein was analyzed for FTL by western blotting using β-actin as a loading control. The mRNA expression was analyzed for FTL gene by RT-PCR. The bars show mean ± SEM, each from three independent experiments. *P<0.05 vs. untreated control groups. FTL, ferritin light chain.

MCF-7 cells were exposed to 1 μM doxorubicin for an additional 24 h. The results showed that concurrent inhibition of FTL with FTH increased cytotoxicity in the MCF-7 cells. Therefore, inhibition of FTL and FTH sensitizes MCF-7 cells to doxorubicin.

Ferritin and doxorubicin effects on ROS formation in MCF-7 cells. To establish the mechanism of action by which ferritin sensitizes MCF-7 cells to doxorubicin, we monitored the intracellular accumulation of ROS using the H_2DCFDA-enhanced chemiluminescence method. Doxorubicin- and ferritin-treated cells showed significantly increased ROS production (Fig. 4A and B), which were particularly high for the doxorubicin-treated groups. Doxorubicin induced intracellular ROS production in MCF-7 cells in a dose-dependent manner. For the combined treatment, ferritin abolished the ROS formation that had been induced by doxorubicin (Fig. 4C).

Ferritin and doxorubicin alter p21Cip/WAF1 protein in MCF-7 cells. To establish whether combined doxorubicin and ferritin treatment mediated the cell cycle pathway, p21Cip/WAF1 was quantified. This is a protein related to the cyclin-dependent kinase inhibitor 1 or CDK-interacting protein 1 for cell proliferation inhibition. The combined drug treatment reduced the levels of the p21Cip/WAF1 protein compared to the doxorubicin treatment alone (Fig. 5). Treatment with ferritin did not only induce p21Cip/WAF1 protein but slightly reduced the protein expression compared to the control. The combination of ferritin and doxorubicin caused a reduction in p21Cip/WAF1 and was associated with a marked proliferative effect in MCF-7 cells.

Discussion

Ferritin is a major iron storage protein essential to iron homeostasis. Ferritin plays a key protective role against oxidative stress due to its ability to sequester iron (18), and can protect normal and cancer cells. In cancer patients, elevated levels of ferritin are detected in the serum, and very high levels correlate with aggressive disease and poor clinical outcome (18). In this study, we investigated how ferritin affects cell proliferation and cell death in the doxorubicin-treated cells of the MCF-7 breast cancer line. Our results show that ferritin plays a critical role in breast cancer cell proliferation and extracellular ferritin protects cancer cells against doxorubicin. Additionally, inhibition of ferritin by FTL and FTH siRNA sensitizes MCF-7 cells to anticancer agents. Inhibition of doxorubicin-mediated cell death occurs probably due to a reduction in intracellular ROS formation, leading to reduced p21, a potent cyclin-dependent kinase inhibitor.

Ferritin is overexpressed in many malignancies including hepatocellular carcinoma (19), Hodgkin's lymphoma (15), pancreatic cancer (3), and breast cancer (10,17). Serum ferritin is elevated in breast cancer patients and surgical resection of

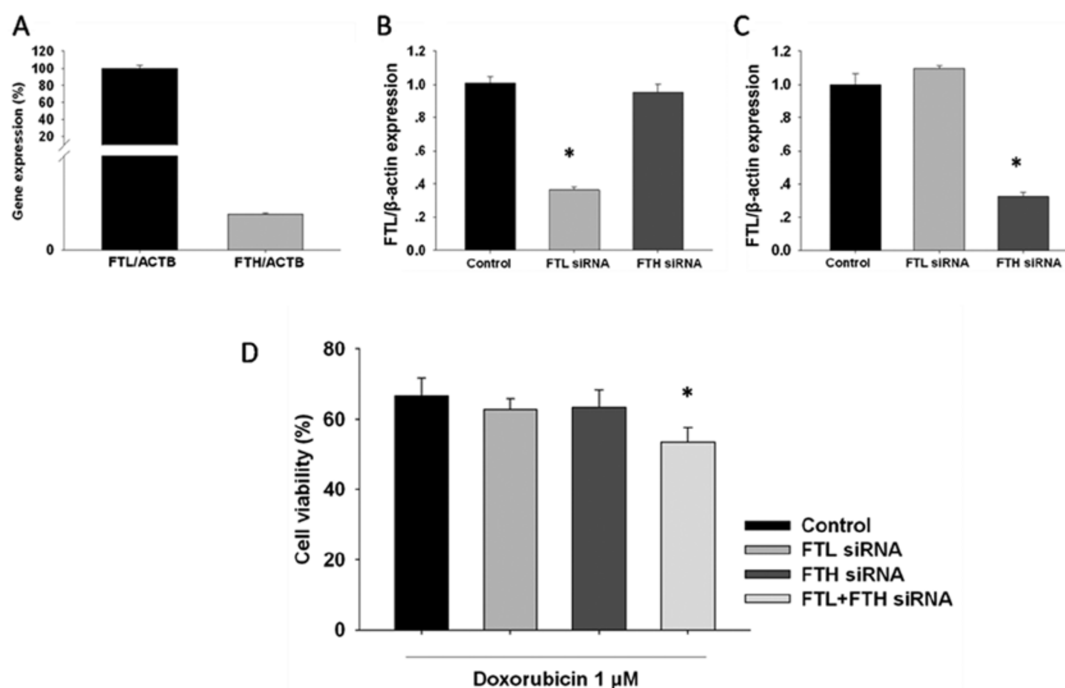

Figure 3. Effects of siRNA knockdown of FTL and FTH on the sensitivity of MCF-7 cells to doxorubicin. The basal levels of FTL and FTH mRNA expression in MCF-7 cells are shown (A). MCF-7 cells were transfected with siRNA against FTL and FTH for 24 h and total RNA was prepared and analyzed by RT-PCR (B and C). The cytotoxicity of doxorubicin in siRNA-treated MCF-7 cells was determined. After transfection for 24 h, MCF-7 cells were treated with 1 μM doxorubicin for another 24 h. The cell viability was evaluated by MTT assay (D). Data are the mean ± SEM, each from three separate experiments. *P<0.05 vs. doxorubicin treatment. FTL, ferritin light chain; FTH, ferritin heavy chain.

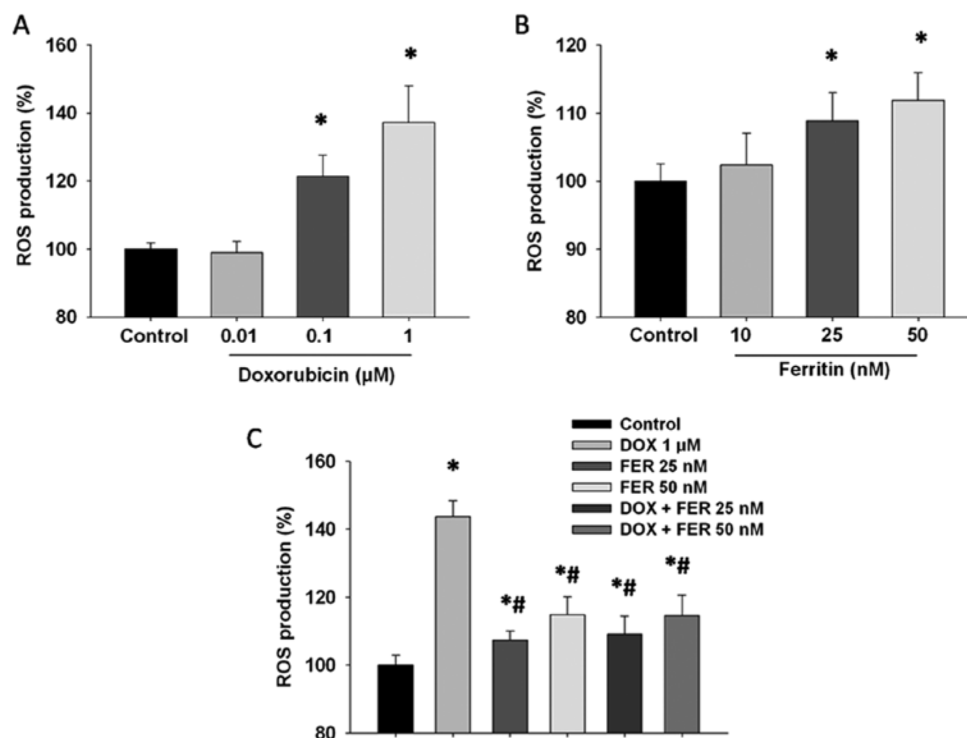

Figure 4. Effects of ferritin on doxorubicin-induced ROS production in MCF-7. Cells (2.5x10^{4} cells/well) were seeded in black 96-well culture plates for 24 h (A and B). Subsequently, cells treated with 1 μM of doxorubicin, 25 and 50 nM of ferritin, and combined treatment for 90 min were assessed for ROS production using 10 μM H_2DCFDA (C). The bars show mean ± SEM, each from three independent experiments. *P<0.05 vs. untreated control groups; #P<0.05 vs. doxorubicin groups. ROS, reactive oxygen species.

tumors lowers serum ferritin levels by ~50% (6). However, the source of serum ferritin elevation has not been firmly established. Previous *in vivo* studies have argued that serum ferritin is primarily derived from macrophages and not

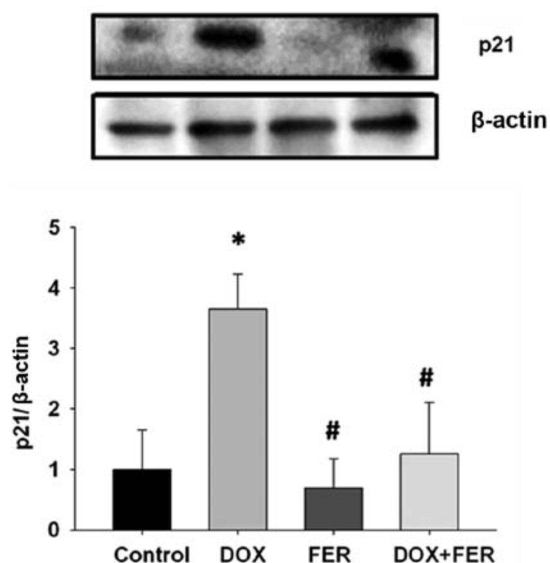

Figure 5. Effects of ferritin and doxorubicin on protein expression with regard to cell proliferation. The western blot analysis shows p21Cip/WAF1 protein expression in MCF-7 cells following treatment with the test compounds. Cells were seeded in a 100-mm culture dish for 24 h and starved for 24 h. On day 1, the cells were treated with 25 nM human liver ferritin. On day 2, the cells were exposed to 1 μM doxorubicin for 24 h. The levels of p21 were normalized using β-actin as a loading control. The bars show the mean ± SEM, each from three independent experiments. *P<0.05 vs. untreated control groups; $^{\#}$P<0.05 vs. doxorubicin groups.

hepatocytes (10,12,21). Higher levels of serum ferritin also induce cancer cell proliferation and resistance to anticancer drug treatment. Ferritin significantly stimulated the proliferation of the T47D and MCF-7 breast cancer cell lines (10). This proliferative effect was iron-independent of ferritin. In our study, human liver ferritin activated the breast cancer cells in a dose- and time-dependent manner. Ferritin also reduced the sensitivity of breast cancer cells to doxorubicin in a dose-dependent manner, consistent with findings showing that increased serum ferritin was associated with poorer outcome in breast cancer patients undergoing chemotherapy (10).

Mounting evidence has shown the proliferative effects of ferritin in many types of cancer including breast cancer (10) and HeLa cancer cells (11). Furthermore, FTL was found to be an independent predictor for breast cancer and present in TAMs (16). In another study, overexpression of L-ferritin, but not H-ferritin, increased proliferation in HeLa cells without affecting intracellular iron levels (11). Ferritin was absorbed by breast cancer cells in a temperature-dependent manner indicating a direct interaction. The proliferative effect of ferritin is apparently independent of iron because apoferritin (iron-poor ferritin) has similar effects to holoferritin (iron-rich ferritin) (10). Our results show that human liver ferritin is absorbed by MCF-7 cells in a time-dependent manner. The results also show that doxorubicin significantly induces human liver ferritin in MCF-7, but that ferritin and doxorubicin do not alter FTL expression in breast cancer cells.

L-ferritin complexes can directly interact with breast cancer cells and stimulate proliferation independently of iron. The proliferative effects of ferritin remain to be elucidated. One study has shown that addition of L-ferritin complexes to primary rat hepatic stellate cells leads to an increase in the phosphorylation of IKK α/β and subsequent activation of NF-κB transcription factor (22). This may activate cell proliferation. TAMs are rich in ferritin and this has led to the hypothesis regarding the contribution of ferritin in cancer biology (16). An elevated expression of ferritin in TAMs may protect them from iron-induced damage and stimulate proliferation (11). For example, L-ferritin levels are 6-fold higher in breast cancer compared to benign breast tissue (10,16). This increase correlated with proliferation, histopathological dedifferentiation and shorter survival. This mechanism may promote cell proliferation and tumor growth. The elevation in serum ferritin is partly due to localized release from TAMs and directly activated cancer cell proliferation.

In the present study, we investigated how ferritin inhibition induces the sensitization of MCF-7 cells to doxorubicin. MCF-7 cells were treated with FTL and FTH siRNA. Of note is that ferritin plays an important role in cancer cell viability especially under severe stress conditions. Due to its antioxidant activity, ferritin has a protective effect against oxidative stress. Our study reveals that FTL and FTH silencing significantly sensitized breast cancer cells to doxorubicin. Inhibition of FTL and FTH by siRNA may result in a significant increase in ROS formation and directly lead to cancer cell death. Doxorubicin treatment alone highly increases ROS levels in MCF-7 cells, while ferritin alone weakly stimulates ROS formation. In the combined treatment, ferritin reduced ROS production by doxorubicin suggesting ferritin may sequester iron that is in released in a particular manner by doxorubicin treatment.

It has been suggested that doxorubicin-induced apoptosis occurs due to the formation of ROS derived from redox activation of doxorubicin. As ferritin acts as an antioxidant, it may decrease the efficacy of doxorubicin as well as other chemotherapeutic drugs (alkylating agents and anthracyclines) whose cytotoxicity is attributable to the production of ROS and the induction of oxidative stress (22). Notably, ROS is capable of inducing cell death via p53-dependent and -indepdent mechanisms (9,23). The results of our study have shown that ferritin reduced the levels of p21, a p53-dependent downstream gene product, and a potent cyclin-dependent kinase inhibitor. Treatment with doxorubicin alone significantly induced p21 expression, while ferritin weakly reduced p21 protein. This is consistent with the strong protective effect ferritin confers on MCF-7 cells to doxorubicin treatment. The mechanism by which ferritin inhibition sensitizes cancer cells to doxorubicin merits further investigation.

In conclusion, ferritin plays an important role in cell proliferation in breast cancer cells. We have demonstrated that ferritin exhibits proliferative activity in MCF-7 cells by promoting cell growth, inhibiting doxorubicin-induced ROS formation, and suppressing the p21Cip/WAF1 expression. A high level ferritin expression has been previously reported in the serum of breast cancer patients. Taken together, these findings suggest that the targeted suppression of ferritin may be a useful strategy to overcome drug resistance in breast cancer chemotherapy. Therefore, ferritin is a potentially useful prognostic factor and candidate drug target for the treatment of breast cancer patients in the future.

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